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13. ABSTRACT ***(Maximum 200 Words)***
Apoptosis, a cell-suicide process required for normal brain development, can also be aberrantly triggered in certain neurodegenerative diseases and following exposure to neurotoxins. We hypothesize that certain components of the signaling pathways activated by these different physiological and pathophysiological stimuli might be shared and could serve as targets for the development of therapeutic approaches. In our application, we proposed to compare the signaling pathways activated by four different apoptotic stimuli using cultures of rat cerebellar granule neurons with the goal of identifying common signaling molecules. During the first three years, our goal was to use one of these apoptotic stimuli - potassium (K+) deprivation - and examine the role of four different apoptosis-regulatory molecules. Results obtained over the past year have strengthened our contention that NF-κB is a molecule central to neuronal survival. Other results have shown that the second molecule under study, p38 MAP kinase, affects neuronal survival in a complex manner. Whereas p38-α might promote neuronal death, its activity might be neutralized by p38-β which promotes survival. Apoptosis by K+ deprivation may be due to the decreased p38-β activity. Finally, our experiments with caspases indicate that these molecules may not be central to the regulation of neuronal apoptosis.

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4. INTRODUCTION

Apoptosis is a cell-suicide process that is required for the normal development of the nervous system. Aberrant and inappropriately regulated apoptosis can, however, lead to undesirable neuronal loss such as that seen in certain neurodegenerative diseases. Apoptosis can also be induced in various neuronal populations by chemical and biological neurotoxins. The intracellular pathways by which these different physiological and pathophysiological stmuli cause neuronal death has not been characterized. We hypothesize that certain components of the signaling pathways activated by different apoptotic stimuli might be shared. Our expectation is that once identified, such molecules could serve as ideal targets for the development of approaches to protect or treat individuals against the actions of neurotoxic agents. In our grant application, we had proposed to compare the signaling pathways activated by four different apoptosis-inducing stimuli using cultures of cerebellar granule neurons from the rat brain with the goal of identifying common molecular components. Specifically, we proposed to induce apoptosis in neuronal cultures by (i) potassium (K+)-deprivation, (ii) treatment with the environmental neurotoxin, methyl mercury, (ii) treatment with b-amyloid protein (βAP) accumulation, which has been implicated in Alzheimer's disease, and (iv) overstimulation with the neurotransmitter, glutamate. We proposed to examine the involvement of four signaling molecules - NF-κB, p38 MAP kinase, caspases, and mGluR4 - in the regulation of cell-death by the different apoptotis-inducing stimuli.

5. BODY

A. Research Accomplishments:

During the first three years we had proposed to examine whether four molecules - NF-κB, p38 MAP kinase, caspases, and mGluR4 - are causally involved in the regulation of low K+ (LK)-mediated neuronal apoptosis. During the past year, we have made substantial progress on one of these molecules - NF-κB - and have obtained valuable information on two others - p38 MAP kinase and caspases. Our results are described below:

NF-κB

1. In our proposal, we had described that SN-50, an inhibitor of NF-κB, inhibited the survival effects of high K+ (HK). We had proposed to complement these results using two additional inhibitors of NF-κB activity - TPCK and PDTC (pg. 22). We have performed these experiments and have found that like SN-50, TPCK and PDTC also inhibit HK-mediated survival (Fig. 1).

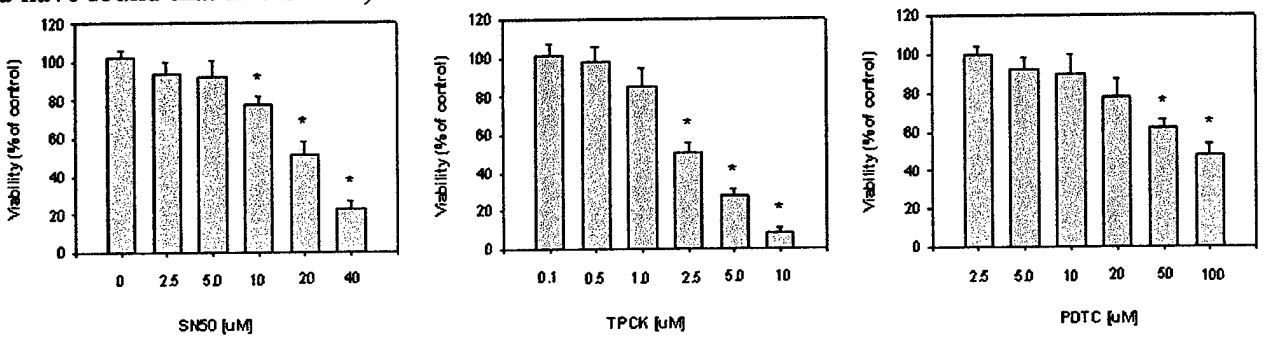

Fig. 1: NF-κB inhibitors induce apoptosis even in the presence of HK.
Effect of inhibitors on survival. Neurons were switched to serum-free culture medium containing HK supplemented with various doses of three NF-κB inhibitors- SN50, TPCK and PDTC. Control cells received HK only. Viability was assayed 24 h later using the MTT assay.

2. We had proposed to overexpress IκB to more directly determine whether NF-κB is involved in HK-mediated survival (pg. 22). We have done this and find that overexpression of IκB-α does inhibit HK-mediated survival indicating that NF-κB is involved in maintaining neuronal survival (Fig. 2).
3. We had proposed to examine if overexpression of RelA could prevent apoptosis caused by K+ deprivation (pg. 22). We find that this is the case using a CMV-RelA vector (Fig. 2).

We have thus completed the experiments concerning the role of NF-κB in low K+ (LK)-induced apoptosis and find that this transcription factor is necessary for neuronal survival. Although not proposed in the grant application, we have followed these results up to examine whether the levels of endogenous IκB was increased or whether RelA expression was reduced by LK-treatment. Very surprisingly neither the levels of IκB-α or of IκB-β was altered by K+ deprivation as judged by Western blot analysis (Fig. 3). Similarly, no alteration was detectable in the expression of endogenous RelA or any of the other four NF-κB proteins (p50, p52, c-Rel, and RelB). Since the activity of p65 can also be increased by its phosphorylation, we examined whether p65 phosphorylation was reduced by K+ deprivation. We find that although phosphorylated to a modest level in HK-medium, K+ deprivation did not cause a reduction in the extent of phosphorylation (Fig. 4). Interestingly, phosphorylation of IκB-β is higher in healthy cultures compared with those induced to die by K+ deprivation (Fig. 4). We are presently examining whether IκB-β phosphorylation does in fact, affect neuronal viability.

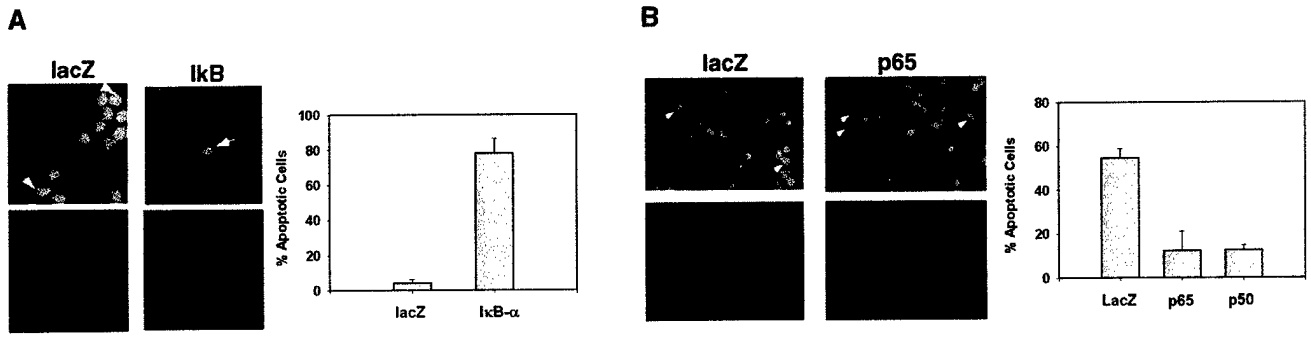

Fig. 2: IκB-overexpression promotes while p65-overexpression inhibits neuronal death. Cultures were transfected with lacZ (as control) or IκB or p65-expressing vectors. Apoptosis (as judged by condensed nuclei) was assayed 24 h after transfection.

A. IκB overexpression. Cultures were tranfected with CMV-lacZ or CMV- IκB and switched to HK-medium for 24h. Immunocytochemical analysis is shown in the left panel. Upper half shows staining with the nuclear stain, DAPI. Lower half shows lac-Z / IκB-immunostaining. IκB-overexpressing neurons are generally apoptotic (condensed nuclei). On the right, is quantification derived from 3 individual experiments performed in duplicate.

B. p65 overexpression. Cultures were tranfected with CMV-lacZ or CMV- p65 and switched to HK-medium for 24h. Immunocytochemical analysis is shown in the left panel. Most p65-expressing neurons shown healthy nuclei. Similar protection was observed using a p50-expression vector.

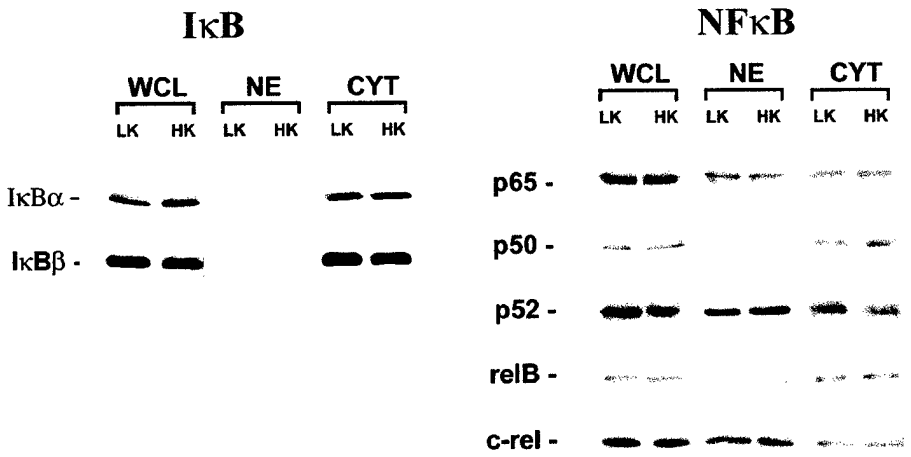

Fig. 3: K+ deprivation does not alter the expression of NFκB and IκB proteins. Expression of IκB and NFκB proteins in whole cell lysates (WCL), nuclear lysates (Nuc), and cytoplasmic lysates (Cyt), prepared from cultures switched to HK or LK medium for 6h.

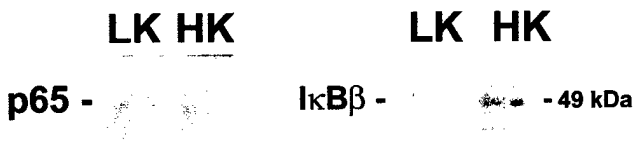

Fig. 4: Phosphorylation of IκB, but not of p65, is reduced by LK treatment. Cultures were switched to LK or HK medium containing 32-P-phosphate. p65 or IκB were immunoprecipitated and analyzed by SDS-PAGE.

In other experiments, we have used SN-50 to determine whether NF-κB might be a convergent point in the regulation of neuronal survival. As seen with HK-mediated survival, we find that inhibition of NF-κB activity in neuronal cultures by SN-50 inhibits the survival-promoting effects of other factors such as IGF-1, cyclic AMP, and lithium (data not shown), consistent with

the idea that NF-κB is a critical component of the pathways involved in the prevention of neuronal apoptosis.

These results have been recently submitted for publication to the Journal of Neuroschemistry. We are awaiting the reviewers' comments.

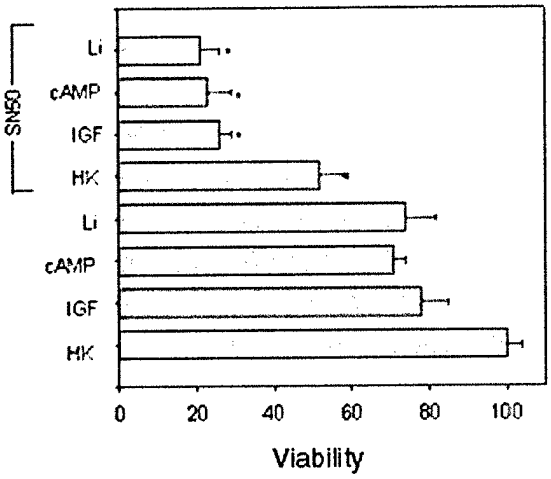

Fig. 5: SN-50 inhibits the survival-promoting effects of different factors. Granule neurons were switched to medium containing HK, IGF-1, forskolin (cAMP) or lithium (Li) in the presence or absence of the NF-κB inhibitor, SN-50. Viability was quantified 24 h later.

p38 MAP kinase

Our hypothesis was that p38 MAP kinase, which has four known isoforms in mammals, is generally proapoptotic in cerebellar granule neurons. This hypothesis was based on preliminary observations that SB203850, an inhibitor of p38-α and p38-β caused cell-death even in HK-medium.

1. Since our pharmacological experiments using SB203580 were only preliminary, we had proposed to confirm these results and to extend them using another p38 inhibitor - SB202190 (pg. 20). Upon detailed analysis we find (in contrast to our preliminary observations) that treatment of neuronal cultures with SB203580 actually protects against LK-induced apoptosis indicating that the activation of one or more of the p38 family members are involved in LK-induced apoptosis. Similar results were obtained using SB202190.

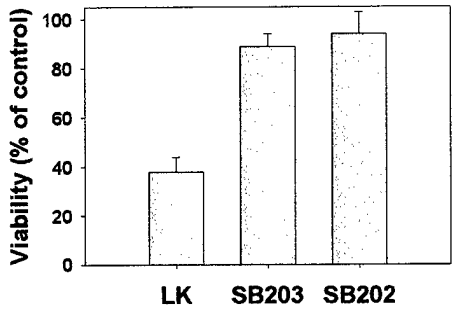

Fig. 6: p38 MAP kinase inhibitors inhibit LK-induced apoptosis Cultures were switched lo LK-medium containing no additives (LK) or supplemented with 20uM SB203580 (SB203) or 20uM SB202190 (SB202). Viability was measured by FDA-staining at 24h after the switch. Control cells received HK-medium.

2. We had proposed to look at the expression of various p38 isoforms in our cultures (pg. 20). Reports by different laboratories indicate that p38-γ and p38-δ are not expressed in the brain. We have therefore focused on the two other isoforms - p38-α and p38-β - both of which are expressed

in the brain and have looked at the expression of these isoforms in cultures switched to HK or LK medium. We find that the levels of p38-α and p38-β are similar in HK and LK medium. This is not surprising given that p38 function is generally regulated at the level of its activity (rather than synthesis), which is dependent on its phosphorylation.

FIG 7

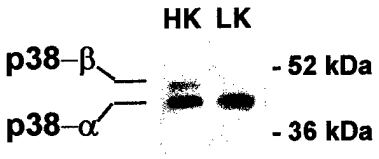

FIG 8

Fig. 7: Levels of p38-α and p38-β are not changed by K+ deprivation. Neurons were switched to HK or LK medium for 4h. Whole cell lysates were prepared and analyzed by Western blotting using a p38 antibody that recognizes both α and β forms (left panel) and an antibody specific for p38-β (right panel).

Fig. 8: Phosphorylation of p38-β is lowered by K+ deprivation. Lysates from cultures treated for 4h in HK or LK medium were immunoprecipitated using a phospho-p38 antibody that recognizes both p38 forms (α and β). The immunoprecipitate was analyzed by Western blotting using an antibody that recognizes both forms.

3. We had proposed to look at the activity of p38 in healthy and apoptotic cultures. This has been done by immunprecipitating phosphorylated p38 using a p38 antibody (reacting against all p38 isoforms). The immunoprecipitate was subjected to Western blotting using a phospho-p38 antibody (which specifically recognizes phosphorylated p38-α and p38-β). As shown in Fig. 8, phosphorylation of p38-β is detectable in HK but is reduced to undetectable levels in LK. In contrast p38-α appears to be active in both HK and LK conditions.

4. We had proposed to overexpress specific p38 isoforms. We started to do this using CMV-driven expression vectors for p38-α and p38-β (provided to us by Dr. J. Han, Scripps Inst., CA),. Based on a single transfection experiments we find that overexpression of p38-α induces apoptotic death in cultures kept in HK-medium (data not shown). Overexpression of p38-β has no such proapoptotic effect. On the contrary, overexpression of p38-β reduces the amount of cell-death caused by K+ deprivation (data not shown). We hope to confirm these results very soon. Furthermore, we have obtained vectors expressing dominant-negative (dn) of p38-α and p38-β. Our expectation is that overexpression of dn-p38-α will prevent LK-induced apoptosis whereas overexpression of dn-p38-β will inhibit HK-mediated neuronal.

Taken together, our results are consistent with the possibility that p38-α and p38-β have opposing effects on neuronal viability; p38-β is neuroporotective whereas p38-α is proapoptotic. In the presence of p38-β the apoptotic effect of p38-β is neutralized. In LK-medium, however, p38-β activity is reduced allowing p38-α to induce apoptosis. This action of p38-α can be inhibited by SB203580 or SB202190, which then leads to increased survival. We hope to confirm these findings in the near future.

Caspases

In our proposal, we had presented evidence indicating that in neuronal cultures from the rat, caspase inhibition could be inhibited by the pan-caspase inhibitor, Boc.Asp.fmk, and to a partial

extent by the caspase-3 / 7 sensitive inhibitor, DEVD.fmk. However, our results ruled out caspase-3, itself, as being necessary for LK-induced neuronal death. Similar results were obtained by other labs as cited in our proposal. Since submission of the grant application, we examined the role of caspases in neuronal death more closely. Interestingly, we find that in contrast to rat cultures, treatment of murine cultures with general and specific caspase inhibitors had no inhibitory effect on neuronal death. A similar failure of caspase inhibitors to prevent LK-mediated death of cerebellar granule neurons has also been reported by other labs (Llyod Greene's lab, Columbia University, and Eugene Johnson's lab, Washington University School of Medicine). The need for caspases in some cases but not in others may reflect species and strain-specific differences. It appears, however, that caspases are not generally necessary for LK-induced apoptosis and therefore cannot represent a critical convergent point in the signaling pathways activated by different apoptosis-inducing stimuli.

B. Training Accomplishments:

Funds from the grant were used for the salary of a postdoctoral fellow, Sunitha Kumari, who joined my lab in April 2000. Sunitha came to us with excellent credentials and research experience. She was previously a postdoctoral fellow at the University of North Texas Medical Center, Fort Worth, and obtained her Ph.D. from Texas A & M University, College Station. Since joining my lab, Sunitha has been working on p38 MAP kinase and has been investigating its role in the regulation of apoptosis. Funds from the grant were also used to support a second-year graduate student, Lucy Liu, as a Graduate Research Assistant in the Spring semester, 2000. In collaboration with Sunitha, Lucy has obtained some interesting results which are being included in a manuscript that is being prepared. This semester (Fall 2000), a Research Assistantship has been provided to a first-year graduate student, Kyle Johnson. Kyle has contributed results that have been included in the NF-κB manuscript currently being reviewed. Both students and the postdoctoral fellow have gained tremendously from the training made possible by the grant and are likely to make important research contributions.

7. APPENDIX

1) Key research accomplishments

- NF-κB is required for the survival effects of HK as well as other survival-promoting factors.
- Caspases may not be a critical component of the signaling pathways activated by different apoptosis-inducing stimuli.
- At least one of the four p38 MAP kinase isoforms is involved in promoting neuronal apoptosis.

2) List of reportable outcomes

- Manuscript: Koulich E, T. Nguyen T, Johnson K, D'Mello SR. NF-κB is involved in the survival of cerebellar granule neurons: Association of IkB-b phosphorylation with cell survival. J. Neurochem. (Submitted).
- Abstract: Koulich E, Nguyen T, D'Mello, SR. Soc. Neuroscience Annual Mtg., New Orleans, LA (Nov.4 to 9, 2000).
- Funding: Based on the results obtained over the past year, we have submitted a R01 grant application to the NIH. The grant to the NIH shares no overlap with the grant funded by the USAMRMC.
- Training: Funds from the grant were used to support two graduate students (1 semester each). In addition, the salary of a postdoctoral fellow comes out of the grant.

3) Conclusions

Our results are consistent with a central role for NF-κB in the inhibition of neuronal apoptosis. The role of p38 MAP kinases is complex and may involve opposing activities of different isoforms. Caspases may not be a central component of the apoptotic pathways triggered by different cell-death inducing stimuli.

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Topic 1: 17. Neuronal death

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Abstract Title: NF-κB MAINTAINS SURVIVAL OF CEREBELLAR GRANULE NEURONS

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Key words: APOPTOSIS, p65, I kappa B, Potassium deprivation

Abstract: The NF-κB transcription factor consists of dimeric complexes belonging to the Rel family, which includes p50, p52, p65 (relA), c-rel, and relB. NF-κB activity is tightly controlled by IκB proteins which bind NF-κB preventing its translocation to the nucleus. Activation of NF-κB is most often mediated by IκB degradation which thus permits NF-κB translocation. We have investigated the role of NF-κB in cultured cerebellar granule neurons. We find that survival of these neurons induced by high potassium (HK) medium is blocked by 3 separate inhibitors of NF-κB activity - SN-50, TPCK, and PDTC, suggesting that NF-κB activity is required for HK-mediated survival. EMSA assays reveal 2 major complexes that bind to the NF-κB binding site in HK medium. The level of the larger of the two complexes declines following potassium (K+) deprivation, a stimulus that induces neuronal apoptosis. Supershift assays indicate that this complex contains p65, which implies that reduction of p65 activity may be involved in the induction of apoptosis. Consistent with this idea, overexpression of p65 by transfection of a p65-expressior vector prevents neuronal death caused by K+ deprivation whereas overexpression of IκB-α induces cell death even in the presence of HK . Surprisingly, however, neither the level of endogenous p65 or that of IκB-α is altered by K+ deprivation as judged by immunoblot analysis of whole cell lysates and nuclear extracts. Similarly, no changes in the level of p50, p52, c-rel, or relB are seen. Since neithe levels of NF-κB proteins or IκB are altered, promotion of neuronal survival by NF-κB may occur by other IκB-independent mechanisms. Such mechanisms are currently being explored.

Supported by: The Department of Defense

NF-κB is involved in the survival of cerebellar granule neurons: Association of IKBβ phosphorylation with cell survival.

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Abbreviations used: IKK, IκB kinase; HK/LK, high/low potassium; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolim bromide; NGF, nerve growth factor; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone; PDTC, pyrrolidinedithiocarbamate

Key words: NF-κB, IκB, cerebellar granule neurons, apoptosis, neuronal survival

RUNNING TITLE : *NF-κB IN NEURONAL SURVIVAL*

The NF-κB transcription factor consists of dimeric complexes belonging to the Rel family, which includes p50, p52, p65 (RelA), RelB and c-Rel. NF-κB activity is tightly controlled by IκB proteins which bind to NF-κB preventing its translocation to the nucleus. Activity of NF-κB is most often mediated by IκB degradation, which permits NF-κB to enter the nucleus. We have investigated the role of NF-κB in the survival of cerebellar granule neurons. We find that survival of these neurons in high potassium (HK) medium is blocked by three separate inhibitors of NF-κB activity: SN50, N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), and pyrrolidinedithiocarbamate (PDTC), indicating that NF-κB is required for neuronal survival. Gel shift assays reveal three complexes that bind to the NF-κB binding site in HK medium. Switching of these cultures to low potassium (LK) medium, a stimulus that leads to apoptotic death, causes a reduction in the level of the largest complex, which contains p65. Overexpression of p65 by transfection inhibits LK-induced apoptosis whereas overexpression of IκBα promotes apoptosis even in HK-medium. Surprisingly, however, neither the level of endogenous p65 or that of IκBα and IκBβ is altered by LK treatment. Similarly, no changes are seen in the nuclear or cytoplasmic levels of p50, p52, RelB and c-Rel. Phosphorylation of p65, which can lead to its activation, is unchanged. Phosphorylation of IκBβ is, however, reduced by K+ deprivation. Besides being necessary for HK-mediated neuronal survival, NF-κB is also involved in the survival-promoting effects of IGF-1 and cyclic AMP as judged by the ability of SN50 to inhibit the actions of these survival factors and the ability of these factors to inhibit the K+ deprivation-induced alterations in the DNA-binding activity of NF-κB. Taken together, our results show that NF-κB may represent a point of convergence in the signaling pathways activated by different survival factors and that NF-κB is regulated by uncommon mechanisms in cerebellar granule neurons.

Introduction

The NF-κB family of proteins are ubiquitously expressed and inducible transcription factors that regulate the expression of genes involved in disparate processes such as immunity and inflammation, growth, development, viral-gene transcription and in cell-death regulation (Ghosh et al., 1998, Karin and Ben-Neriah, 2000; Mattson et al., 2000). In mammalian cells, there are five NF-κB proteins, p50, p52, p65 (RelA), RelB and c-Rel. NF-κB is composed of homodimers and heterodimers of these proteins, typically p65 : p50, which are held in the cytoplasm by the inhibitory IκB proteins which bind NF-κB and mask its nuclear localization signal.

The role that NF-κB plays in the regulation of neuronal survival is complex. NF-κB has been found to be involved in the survival promoting effects of NGF and cytokines (Maggirwar et al., 1998; Middleton et al., 2000) and its activation is associated with neuroprotection against death-inducing stimuli such as exposure to β-amyloid protein, oxidative stress, and nitric oxide (Barger et al., 1995; Mattson et al., 1997; Lezoulac'h et al., 1998; Kaltschmidt et al., 1999; for review, Mattson et al., 2000). In some cases, however, NF-κB promotes neuronal death. NF-κB activation is detected *in vitro* following exposure to apoptotic stimuli (Lin et al., 1998; Cheema et al., 1999) and *in vivo* after global ischemia, kianate-induced seizures, and traumatic spinal cord injury (Prasad et al., 1994; Grilli et al., 1996; Clemens et al., 1997; Bethea et al., 1998; for review, Mattson et al., 2000). Furthermore, increased levels of NF-κB activity have been detected in vulnerable regions of the CNS of Alzheimer's, Parkinson's, and amyotrophic lateral sclerosis patients (Hunot et al., 1997; Kaltschmidt et al., 1997; Migheli et al., 1997) raising the possibility that NF-κB may play a causal role in neurological disorders (Kaltschmidt el al, 1993). Whether NF-κB inhibits or promotes apoptosis might depend on the cell type and the nature of the apoptosis-inducing stimulus. Additionally, the composition of the NF-κB homo/heterodimer is likely to play a critical role in regulation of apoptosis. In support of this idea are the observations that p65 activation increases survival in

many cases whereas c-Rel has been associated with apoptosis (Abbadie et al., 1993; Wu et al., 1996; Beg and Baltimore, 1996). It is not clear precisely how NF-κB activation regulates cell survival although induction of the genes encoding the antiapoptic bcl-2 homolog A1, and some members of the inhibitor of apoptosis proteins (IAPs) by NF-κB have been reported (Chu et al., 1997; Wang et al., 1998; Wang et al., 1999).

The molecular mechanisms involved in the activation of NF-κB are beginning to be understood (Ghosh et al., 1998; Karin and Ben-Neriah, 2000). In general, activation of NF-κB is the result of IκB phosphorylation by the multisubunit IκB kinase (IKK) complex, which leads to IκB degradation. This permits NF-κB to translocate to the nucleus and activate transcription of target genes. A number of IκB proteins have been identified including IκBα, IκBβ, IκBγ, and IκBε proteins of which IκBα and IκBβ are the best studied (Karin and Ben-Neriah, 2000). IκBα and IκBβ share a characteristic ankyrin repeat motif required for their interaction with NF-κB. The IKK-mediated phosphorylation that leads to the degradation of IκBα and IκBβ occurs at two serine residues located at the amino termini of these proteins (Karin and Ben-Neriah, 2000). In addition to these sites, IκBα and IκBβ can be phosphorylated by other cellular kinases such as DNA-dependent protein kinase, and casein kinase II at the C termini (Liu et al., 1998; McKinsey et al., 1996). The biological significance of phosphorylation at these other sites is poorly understood. Although activation of NF-κB proteins is generally regulated at the level of IκB-breakdown mediated translocation, the transcriptional activity of p65 can also be increased by phosphorylation (Wang and Baldwin, 1998; Zhong et al., 1998). Furthermore, interaction with the coactivator CREB-binding protein (CBP)/p300 has been found to increase the activity of p65 in some cases (Perkins et al., 1997; Merika et al., 1998).

We have examined the role of NF-κB in the regulation of survival/apoptosis using cultures of cerebellar granule neurons. These neurons can be cultured and maintained *in vitro* if depolarizing levels of potassium (K+) are provided. Lowering of K+ levels induces apoptosis (D'Mello et al., 1993 ; Yan et al., 1994). Apoptosis

induced by K+ deprivation can be prevented by IGF-1 as well as pharmacological agents such as cyclic AMP (D'Mello et al., 1993). Using this paradigm, we show that NF-κB is necessary for neuronal survival and that overexpression of certain NF-κB members can prevent low K+ induced apoptosis. Interestingly, however, neither the levels of IκB within the cytoplasm, or the nuclear expression of the individual NF-κB members are altered following K+-deprivation. LK-induced death is, however, accompanied by a reduction in the phosphorylation of IκBβ.

Materials and Methods

Reagents and plasmids. All reagents were from Sigma unless specified otherwise. SN-50 was purchased from Calbiochem. IGF-1 was from Roche Biochemicals. CMV-lacZ was purchased from Clonetech. FLAG-tagged CMV-p65 and CMV-p50 were kindly provided by Dr. Baldwin (University of North Carolina, Chapel Hill), and myc-tagged IκBα by Dr. Richard Gaynor (University of Texas Southwestern Medical Center).

Cell culture and treatments. Granule neuron cultures were obtained from dissociated cerebella of 7-8 day old rats as described previously (D'Mello et al., 1993). Cells were plated in Basal Eagle's Medium with Earles salts (BME) supplemented with 10% fetal calf serum (FCS), 25 mM KCl, 2 mM glutamine (Gibco-BRL), and 100 ug/ml gentamycin on dishes coated with poly-L-lysine in 24-well dishes at a density 0.5 x 10^6 cells/well or 2.5 x 10^7 cells/100 mm dish. Cytosine arabinofuranoside (10 uM) was added to the culture medium 18 - 22 h after plating to prevent replication of nonneuronal cells.

Unless indicated otherwise, cultures were maintained for 6 - 7 days prior to experimental treatments with IGF-1 or pharmacological agents. For this, the cells were rinsed once and then maintained in low K^+ medium (serum-free BME medium, 5 mM KCl) with or without the agents, or in the case of control cultures, in high K^+ medium (serum-free BME medium, supplemented with 20 mM KCl). Treatment of cultures with NF-κB inhibitors was initiated 30 min prior to rinsing and was maintained through the subsequent incubation in LK medium.

Neuronal survival. Neuronal survival was quantified by the MTT assay as described by Kubo et al.,1995. Briefly, the tetrazolium salt MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolim bromide] was added to the cultures at a final concentration of 1 mg/ml, and incubation of the culture was continued in the CO_2 incubator for a further 30 min at 37°C. The assay was stopped by adding lysis buffer [20% SDS in 50% N,N-dimethyl formamide , pH 4.7]. The absorbance was

measured specrophotometrically at 570nm after an overnight incubation at room-temperature. The absorbance of a well without cells was used as background and subtracted.

Western blotting. For whole-cell lysates, the culture medium was discarded, the neurons washed twice with cold phosphate-buffered saline (PBS), and lysed in sodium dodecyl sufate (SDS) - polyacrylamide gel electrophoresis buffer [62.5 mM Tris-Cl, pH 6.8, 2% SDS, 5% glycerol, 1% 2-mercaptoethanol, and bromophenol blue]. Following heating at 95^0 for 5 min, proteins were subjected to SDS-polyacrylamide gel electrophoresis and transferred electrophoretically to polyvinylidene difluoride membrane (Millipore). After staining with Ponceau S (Sigma) to verify uniformity of protein loads / transfer, the membranes were analyzed for immunoreactivity. Incubation with primary antibodies were performed overnight at 4^0C and with secondary antibodies for 1h at room-temperature. Immunoreactivity was developed by enhanced chemiluminescence (ECL; Amersham) and visualized by autoradiography.

The following primary antibodies were used : p65 and p52 (Santa Cruz Biotech.; 1 : 1000 dilution), p50 (Santa Cruz Biotech., 1:50), c-Rel and RelB (Santa Cruz Biotech., 1 : 500), IκBα and IκBβ (Santa Cruz Biotech.; 1 : 500). Secondary antibodies were peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz, 1:10,000) and donkey anti-goat IgG (Santa Cruz, 1:5,000).

Transfection. Granule neuron cultures plated on glass coverslips in 24-well dishes and transfected 5 or 6 days later using the calcium-phosphate protocol of Dudek et al., 1998. Briefly, the cells were washed twice with serum-free Dulbecco's Modified Eagle medium (DMEM) (Gibco-BRL) and incubated for 37^0C in a CO2-incubator for 1 h. The conditioned-medium containing serum and HK was kept aside. For each well a mixture was prepared as follows - 15 ul of HeBS buffer was combined with 15ul of a 0.25M CaCl2-solution containing 3 ug of plasmid DNA, vortexed, and allowed to sit at room temperature for 30 min. The mixture was added to the cells

dropwise and allowed to incubate for 30 min. After washing of the cells twice with DMEM, the cultures were fed with the conditioned medium in which they had been maintained after plating. The following morning, the medium was replaced with HK or LK medium (as described in the text and legends) for a period of 24 h, unless indicated otherwise.

Immunofluorescence analysis of transfected cultures. Immunofluorescence analysis of transfected cultures were performed as follows. The cells were fixed for 15 min in 4% paraformaldehyde. After blocking in 0.1 M phosphate buffer for 30 min, immunostaining was performed by incubation with the primary antibody for 1 h and the secondary antibody for 30 min at room-temeperature. The following mouse monoclonal primary antibodies were used: anti-FLAG (1 : 200, Sigma), anti-myc (9E10, 1 : 200, Santa Cruz Biotechnology, CA) and anti-β-galactosidase (1 : 40,Gibco-BRL). The secondary antibody for all experiments was a Texas Red-conjugated goat anti-mouse antibody (1 :200 , Jackson ImmunoResearch Labs). During washing of the secondary antibody, nuclei were stained in 4,6-Diamidino-2-phenylindole (1 ug/ml) for 5 min at room-temperature. Coverslips were mounted with with fluormount (Molecular Probes), viewed with a Olympus Optiphot-2 microscope under a 40X objective and images captured with a SPOT cooled CCD-camera. Apoptotic cells were recognized by bright staining and condensed/fragmented nuclear morphology when viewed under ultraviolet light (260nm).

DNA fragmentation analysis. Fragmentation of DNA was analyzed as previously described (D'Mello et al., 1993). Soluble DNA was isolated from equal numbers of plated cells (2.5 x 10^7) by cell lysis and elimination of nuclei. After treatment with RNAse A (50 ng/ml, 37^0C for 30 min), the samples were subjected to electrophoresis in a 1.5% agarose gel and the DNA visualized by ethidium bromide staining.

Analysis of p65 and IκBβ phosphorylation. 60mm dishes of 6-7-day old neurons were washed twice with warm, phosphate-free DMEM containing 25mM KCl and incubated overnight in the same medium. The cultures were then incubated for 6h in medium containing [^{32}P] orthophosphate (400μCi; ICN) in either 25mM (HK medium) or 5mM KCl (LK medium). Following lysis in ice-cold RIPA buffer [50mM Tris, pH7.4, 150mM NaCl, 1%NP-40, 0.25% sodium deoxycholate, 0.1% SDS, 1mM Na3VO4, 1mM NaF, 1mM PMSF, 1mM EDTA, 100uL/10^7 cells/mL protease inhibitors cocktail], the lysates were centrifuged for 10 min at 10,000 x g at 4°C. Supernatants were incubated overnight with primary antibody (1.5 - 2ug) and then for 2h with 20ul Protein A/G PLUS-Agarose (Santa Cruz Biotech.). Immunoprecipitates were collected by centrifugation at 2,500 rpm for 5 min at 4°C and pellets resuspended in electrophoresis sample buffer and subjected to SDS-polyacrylamide gel electrophoresis. The gel was dried and analyzed by autoradiography.

Results

Inhibitors of NF-κB activity induce neuronal death

As a first step towards examining whether NF-κB is involved in the regulation of neuronal survival, we examined the effect of SN50, a cell-permeable recombinant peptide that blocks translocation of NF-κB to the nucleus. Previous work has shown than SN50 has no effect on IκB-degradation (Lin et al., 1995) or on the activity of several other transcription factors including AP-1, CREB, and OCT (Maggirwar et al., 1998). As shown in Fig. 1A, SN-50 treatment caused a dose-dependent increase in cell death in cultures maintained in HK (Fig.1A). Substantial loss of viability is observed at 10uM, a dose at which this drug is known to significantly inhibit NF-κB translocation (Lin et al., 1995). To verify that SN50 was not non-specifically toxic, we performed DNA fragmentation analysis of cells treated with the inhibitor. As shown in Fig. 1B, cells treated with SN50 in HK-medium displayed DNA fragmentation, a characteristic feature of apoptotis. In the absence of HK, survival of cerebellar granule neurons can be maintained by IGF-1 and cyclic AMP. As shown in Fig.1C, treatment with SN50 also abolished the survival-promoting activity of IGF-1 and cyclic AMP suggesting that NF-κB may represent a point of convergence in the signaling pathways activated by different survival factors.

While SN-50 has been shown to be a selective inhibitor of NF-κB translocation and has been widely used, an effect of this peptide on some other intracellular target cannot be completely ruled out. Two other commonly used inhibitors of NF-κB are the protease inhibitor, N-tosyl-L-phenylalanine chloromethyl ketone (TPCK; Henkel et al., 1993; Wu et al., 1996; Phillips and Ghosh, 1997) and the antioxidant pyrrolidinedithiocarbamate (PDTC; Shreck et al., 1992; Martinez-Martinez, 1997; Mahboubi et al., 1998). We used TPCK and PDTC to confirm the results obtained using SN50. As shown in Fig. 1A, both drugs inhibit high K+-mediated neuronal survival at doses at which they inhibit NF-κB activity.

DNA-binding activity of NF-κB is altered in neurons primed to die

To examine whether K+ deprivation altered the DNA-binding activity of NF-κB, we used electrophoretic mobility gel shift assays (EMSA). Nuclear extracts were prepared from cultures switched to LK medium for 0 - 4 h and incubated with a radiolabeled NF-κB oligonucleotide probe. As shown in Fig. 2A, three major bands are visible in control cells (0 h). Binding of all three complexes is eliminated in the presence of 100-fold molar excess of unlabeled NF-κB probe (lane 'NF') but not an oligonucleotide containing an AP-1 consensus sequence (lane 'AP'), indicating that binding is specific. The intensity of the band corresponding to Complex 1 is reduced to less than 50% of its original level at 4h (Fig. 2A) and remains low even at 6h (not shown). Since commitment to death in granule neurons occurs within the first 4 -6h after K^+ deprivation (Galli et al., 1995, Schulz et al., 1996, Nardi et al., 1997; Borodezt and D'Mello, 1997), the reduction in Complex 1 may be causally involved in the induction of apoptosis. Besides the change seen in Complex 1, the intensity of the band corresponding to Complex 2 is increased after K+ deprivation and this occurs within an hour. To examine whether the reduction of Complex 1 was related to the induction of cell death rather than the lowering of K^+, we performed EMSA assays on extracts prepared from neurons treated with IGF-1 and cyclic AMP in low K+ medium. Both IGF-1 and cyclic AMP can maintain granule neuron survival in the absence of HK (D'Mello et al., 1993). As shown in Fig. 2B, in the presence of these survival factors, the reduction in binding of Complex 1 seen after K+ deprivation was inhibited.

To identify the components of the complexes that bind the NF-κB probe, nuclear extracts were preincubated with antibodies against p50 or p65. As shown in Fig. 2C, incubation with the p50 antibody caused a supershift accompanied by a slight decrease in the intensity of Complex 2 as well as Complex 1. In contrast, the p65 antibody eliminated formation of Complex 1 suggesting that the reduced activity of p65 is involved in the induction of apoptosis.

Overexpression of p65 inhibits while that of IκBα promotes apoptosis.

To examine if overexpression of p65 can prevent LK-induced neuronal death, neuronal cultures were transfected with a p65-expressing vector (CMV-p65) or a β-galactosidase-expressing vector (CMV-lacZ) and switched to LK-medium. Whereas about 50% of the untransfected cells and lacZ-expressing cells displayed condensed / fragmented nuclei after 24 h of K+ deprivation, less than 15% of the p65-expressing cells appeared apoptotic (Fig. 3A and B). As observed with p65, overexpression of p50 also protected granule neurons from LK-induced apoptosis (Fig. 3 B).

Because NF-κB activation is normally regulated by IκB-degradation and increased IκB expression would lead to a higher amount of NF-κB- sequesteration, we examined whether overexpression of IκBα would induce apoptosis under survival-promoting culture conditions. As shown in Fig.3 C and D, over 75% of the neurons transfected with of an IκBα–expressing vector appeared apoptotic in the presence of HK.

Steady-state levels of endogenous IκBα, IκBβ or p65 are not altered in neurons primed to die. Although our transfection experiments showed that p65 and p50 were protective against apoptosis and that IκBα–overexpression induced neuronal apoptosis, these experiments did not reveal whether LK-induced apoptosis was actually due to changes in endogenous IκB and NF-κB levels. To study this issue we performed Western blot analysis of IκB and NF-κB expression 6h after the switch to LK-medium. We and others have shown that by 4 - 6h of K+ deprivation granule neurons are committed to dying and irreversible intracellular degenerative changes such as DNA fragmentation, are underway (Yan et.al., 1994; Galli et al., 1995; Schulz et al., 1996; Nardi et al., 1997; Borodezt and D'Mello, 1997). More importantly, changes in the DNA-binding activity of NF-κB are clearly completed by 4h of K+ deprivation (Fig. 2). As expected, IκBα expression is confined largely to the cytoplasm in cultures maintained in HK (Fig. 4A). Surprisingly, no increase in IκBα level is discernible after K+ deprivation (Fig. 4A). In addition to

IκBα, translocation of NF-κB can be inhibited by IκBβ. Like IκBα , IκBβ levels remained unchanged following LK treatment (Fig. 4A).

Given that p65 has frequently been implicated in the promotion of cell-survival and because DNA-binding of p65 is reduced following K+ deprivation, we examined its expression. No change was discernible in total cellular, nuclear or cytoplasmic p65-immunoreactivity following K+ deprivation (Fig. 4B). To confirm that there were no alterations in the distribution of p65 following K+ deprivation, we performed immunocytochemical analysis. In agreement with the data from Western blots, no difference in the intracellular localization of p65 was detectable between neurons treated with HK and LK-medium (Fig. 4C). As observed for p65, no K+ deprivation-associated changes were seen in the expression of p50, p52, c-Rel, or RelB (Fig. 4B).

The extent of IκBβ but not p65 phosphorylation is changed by K+ deprivation.

Besides increased nuclear translocation, p65 activity can also be increased by its phosphorylation (Wang and Baldwin, 1998; Zhong et al., 1998). It was therefore conceivable that p65 was phosphorylated in healthy neurons and the extent of phosphorylation and hence activity was reduced upon K+ deprivation. However, *in vivo* labeling and immunoprecipitation of p65 revealed that although phosphorylated to a small extent in healthy neurons, the level of phosphorylation is not changed by K+ deprivation (Fig. 5A).

In vivo labeling did reveal, however, that IκBβ is phosphorylated in HK (Fig. 5B). The extent of phosphorylation was reduced to undetectable levels following 6h of K+ deprivation. Similar analysis of IκBα showed no detectable phosphorylation in either LK or HK conditions (not shown).

Discussion

The survival effect of HK on cultured cerebellar granule neurons is inhibited by different pharmacological inhibitors of NF-κB, indicating that NF-κB is necessary for neuronal survival. Indeed, switching of neurons from to LK-medium causes alterations in DNA-binding activity of various NF-κB complexes. Most significantly, a complex containing p65 is reduced at 4h after K+ deprivation. Given that these neurons become committed to death at 4-6h after K+ deprivation (Borodezt and D'Mello, 1997), this finding suggests that LK-induced apoptosis may be due to reduced p65 activity. Consistent with this idea is the finding that overexpression of p65 prevents LK-induced apoptosis. Inhibition of apoptosis by p65 has been demonstrated in many cell types (Bellas et al. 1997; Levkau et al., 1999; Yang et al., 1999; Anto et al., 2000) and mice lacking p65 show increased apoptosis in the liver and die early during embryogenesis (Beg et al., 1996).

The activity of p65 and other NF-κB proteins is normally regulated by IκB proteins by sequestration in the cytoplasm. Elevated expression of IκB thus inhibits activity of NF-κB. Stimuli that activate NF-κB most often do so by inducing phosphorylation of IκB proteins, which triggers their degradation thus allowing NF-κB to translocate into the nucleus (Ghosh et al., 1998; Karin and Ben-Neriah, 2000). Not unexpectedly, therefore, overexpression of IκBα promotes cell-death even in the presence of HK. Intriguingly, however, the level of endogenous IκBα, or that of IκBß is not reduced in HK-treated granule neurons. As observed for these IκB proteins the level of endogenous p65 in nuclear and cytoplamic extracts is not altered by K+ deprivation. To examine whether the decreased DNA-binding activity of p65 caused by LK-treatment was due to an increase in the expression of some other NF-κB protein which could then outcompete p65 for DNA-binding, we analyzed the levels of the other four members of the NF-κB family. No alterations in the expression of p50, p52, RelB and c-Rel were discernible. The absence of any alterations in the expression of NF-κB proteins is consistent with the finding that the cellular levels of IκB proteins are not affected by K+ deprivation.

It is known that phosphorylation of p65 can increase its DNA-binding and transcriptional activity. It was, therefore, conceivable that LK-induced apoptosis was caused by decreased p65 phosphorylation, rather than reduced translocation. *In vivo* labeling experiments, however, did not detect any change in the extent of phosphorylation following K+ deprivation. Phosphorylation of p65 has been shown in some cases to promote interaction with the transcriptional coactivator CBP/p300 which leads to increased p65 transcriptional activity (Perkins et al., 1997; Merika et al., 1998). A recent report has shown that in PC12 cells, c-jun and p65 compete for binding to CBP (Maggirwar et al., 2000). Apoptosis in sympathetic neurons and granule neurons is preceded by an induction in c-jun expression and phosphorylation (Estus et al., 1994; Ham et al., 1995, Watson et al., 1998) raising the possibility that reduced p65 binding to CBP may contribute to apoptosis. Although we have detected modest interaction between CBP and p65, this is not reduced following K+ deprivation (data not shown). Other novel mechanisms for the regulation of NF-κB activity have been reported. For example, it is known that p65 can dimerize with non- NF-κB proteins such the Egr-1 transcriptional factor or the transcriptional corepressor AES which then prevent p65 from interacting with target promoters (Chapman et al., 2000; Tetsuka et al., 2000). It is not known whether such mechanisms operate in granule neurons deprived of K+.

NF-κB has also been shown to be involved in NGF-mediated survival of sympathetic neurons (Maggirwar et al., 1998). In contrast to cerebellar granule neurons, degradation of IκBα was higher in sympathetic neurons induced to die by NGF deprivation (Maggirwar et al., 1998). Overexpression of c-Rel was found to inhibit apoptosis caused by NGF deprivation (Maggirwar et al., 1998) although whether endogenous c-Rel expression or translocation is reduced by NGF-deprivation was not examined directly. As observed in granule neurons, p65 expression was not affected by NGF deprivation (Maggirwar et al., 1998).

One alteration that is associated with the induction of apoptosis in granule neurons is the reduced phosphorylation of IκBβ. It is unclear how higher IκBβ phosphorylation might enhance neuronal viability. Since the expression of IκBβ

and that of NF-κB subunits are similar in HK and LK medium, it is unlikely that phosphorylation of IκBß leads to its degradation. In addition to IKK, IκBα and IκBß can be phosphorylated by other cellular kinases within the C-terminus. In the case of DNA-PK-induced phosphorylation, the stability of IκBβ is not affected (Liu et al., 1998). It is not known whether the HK-induced IκBß phosphorylation occurs at the two serine residues phosphorylated by IKK and which causes its degradation, or whether other kinases such as DNA-PK are involved in the HK-induced phosphorylation. Interestingly, DNA-PK has been implicated in the suppression of apoptosis in nonneuronal cell types (Nueda et al., 1999; for review, McConnell and Dynan, 1996) and it is well established that this kinase is cleaved and inactivated by caspases during apoptosis (Casciola-Rosen et al.1995; Han et al., 1996; Song et al., 1996;). It is tempting to speculate that the decreased phosphorylation of IκBß during LK-induced apoptosis may be the result of reduced activity of DNA-PK or a related kinase. In contrast to IκBß, IκBα is not phosphorylated in healthy cultures maintained in HK.

In the absence of HK, the survival of granule neurons can be promoted by IGF-1 or cAMP. The survival-promoting effects of these factors are also inhibited by pharmacological inhibition of NF-κB. Furthermore, the alterations caused in DNA-binding activity of NF-κB complexes seen after K+ deprivation are prevented by IGF-1 and cAMP. Whereas HK causes membrane depolarization leading to Ca2+ influx through L-type voltage gated channels (Gallo et al., 1987; Galli et al., 1995), IGF-1 signals via the IGF-I receptor tyrosine kinase in a pathway involving PI-3 kinase (D'Mello et al., 1997; Dudek et al., 1997; Miller et al., 1997). It is likely that cAMP activates a PKA-dependent pathway but which is independent of Ca2+ influx or PI-3 kinase activation (Crowder and Freeman, 1999; Moran et al., 1999). Our results suggest that NF-κB represents a point of convergence in the signaling pathways activated by these different survival factors.

In summary, although NF-κB activity is necessary for HK-mediated survival of cerebellar granule neurons, the mechanism by which it acts is unclear. In most cell-types and in response to a variety of stimuli, NF-κB activity is regulated by the

breakdown of IκB thus leading to increased levels of NF-κB proteins in the nucleus. Our finding that neither the levels of IκBα or IκBβ, nor those of the various NF-κB proteins are affected by K+ deprivation argues against this mechanism of regulation. Similarly, no change in the extent of p65 phosphorylation or interaction of p65 with CBP/p300 is detectable after K+ deprivation. It is possible that phosphorylation of IκBβ is protective for granule neurons and that the reduction of phosphorylation seen after the switch to LK is causally involved in the triggering of apoptosis. Taken together, our results suggest that uncommon and possibly novel mechanisms might be involved in NF-κB –mediated survival of granule neurons.

Figure Legends

Figure 1.

NF-κB inhibitors induce apoptosis.

A. Effect of NF-κB inhibitors on survival. Seven-day old neuronal cultures were switched to serum-free culture medium containing HK supplemented with various doses of three NF-κB inhibitors: SN50, TPCK and PDTC. Viability was assayed 24 h later using the MTT assay. Survival is shown as a percentage of viability of control cells that received HK medium. Results shown come from three independent experiments performed in duplicate. * $p < 0.01$.

B. SN50 treatment causes DNA fragmentation. Cultures were treated with HK, LK, or HK medium containing 20uM SN50. Soluble DNA was isolated 24h later and subjected to agarose gel electrophoresis. M is a molecular weight marker.

C. SN50 inhibits the survival-promoting effects of IGF-1(25 ng/ml), forskolin (cAMP; 10 uM), and lithium (Li, 10 mM) in the ansence or presence of SN50 (20 uM). Viability was assayed 24h later using the MTT assay. * $p < 0.01$.

Figure 2.

EMSA assays of NF-κB-binding.

A. Nuclear extracts were prepared from neurons switched to low K+ medium for 0, 1, 2 and 4 h. Extracts were used in EMSA assays using radiolabeled NF-κB probe. 3 complexes (labeled 1, 2 and 3) can be observed. All three complexes are eliminated in the presence of 100-fold excess of unlabeled NF-κB-oligo (lane "NF") but not with an oligo containing an AP-1 binding site (lane "AP") demonstrating specificity of binding. Complex 1 is reduced at 4h.

B. Alterations in DNA-binding pattern of NF-κB proteins are inhibited by survival factors. Lysates were prepared from cells switched for 4 h to low K+ (LK) medium with no additives or supplemented with high K+ (HK), IGF-1 (IGF) or forskolin (cAMP).

C. Complex 1 contains p65 while complex 2 contains p50. Nuclear extracts were preincubated with antibodies to either p50 or to p65 before binding reactions were performed. p50 antibody reduces intensity of Complex 2 but does not affect Complex 1 significantly. Asterisk shows super-shifted complex. p65 antibody completely eliminates binding to Complex 1 without affecting Complex 2 or 3. Complex 3 is not affected.

Figure 3.

Overexpression of p65 or p50 inhibits while that of IκBα induces cell death. Five-day old granule neuron cultures were transfected with expression vectors using the calcium-phosphate method. Panels A and B refer to p65 and p50 overexpression while panels C and D refer to IκBα overexpression.

A. Neuronal cultures were transfected with expression vectors expressing lacZ, FLAG-tagged p65 or p50. The next morning the cultures were switched to LK medium and viability of transfected cells anayzed 24h later. Upper panels show nuclei stained with DAPI, and lower panels show neurons overexpressing lacZ or p65 as judged by lac Z or FLAG-immunostaining. As seen in the upper panels, a significant proportion of the nuclei appear apoptotic (condensed). The proportion of lacZ-expressing neurons (arrow) that are apoptotic is similar to that of untransfected cells. Neurons expressing p65 (arrow) showed much higher viability than those expressing lacZ.

B. Quantification of results obtained from 3 experiments performed in duplicate. * $p < 0.01$

C. Neuronal cultures were transfected with expression vectors expressing lacZ or myc-tagged IκBα. The next morning the cultures were switched to HK medium and viability of transfected cells anayzed 24h later. Upper panels show nuclei stained with DAPI, and lower panels show neurons overexpressing lacZ or IκBα as judged by lacZ or myc-immunostaining. Nuclei of IκBα-transfected neurons (arrows) appear apopototic as judged by DAPI staining.

D. Quantification of results obtained from 3 experiments performed in duplicate. * $p < 0.01$

Figure 4.

Levels of IκB and NF-κB proteins are not altered in neurons primed to undergo apoptosis.

7-8 day old cultures of granule neurons were switched to HK or LK-medium for 6h and subjected to Western blotting (A and B) or switched for 24h and subjected to immunofluorecence analysis (C). Approximately 10ug, 15 ug, and 10 ug of total protein from cytoplasmic and nuclear extracts, and whole cell lysates, respectively were analyzed per lane.

A. Analysis of IκBα and IκBβ expression. Whole cell lysates (WCL) or nuclear and cytoplasmic extracts (NE and CYT, respectively) were prepared and expression investigated by Western blot analysis using antibodies specific for IκBα and IκBβ

B. Analysis of p50, p52, p65, c-Rel, and RelB expression. Whole cell lysates (WCL) or nuclear and cytoplasmic extracts (NE and CYT, respectively) were prepared and expression investigated by Western blot analysis.

C. Cultures that wre switched to LK or HK medium for 24h were fixed with 4% paraformaldehyde and the cells subjected to immunofluorescence analysis using a polyclonal anibody against p65. Upper panels show nuclear staining with DAPI, while lower panels show p65 immunoreactivity. Only non-condensed cells are shown.

Figure 5.

IκBβ but not p65 phosphorylation is affected by K+ deprivation.

7-8 day old neuronal cultures were switched to HK or LK medium. Phosphorylated proteins were labeled *in vivo* by the addition of [^{32}P] orthophosphate to cultures at the time of switching. Whole-cell lysates were prepared 6h after the switch, p65 or IκBβ immunoprecipitated and analyzed by PAGE followed by autoradiography. A and B show p65 phosphorylation in HK and LK medium, respectively.

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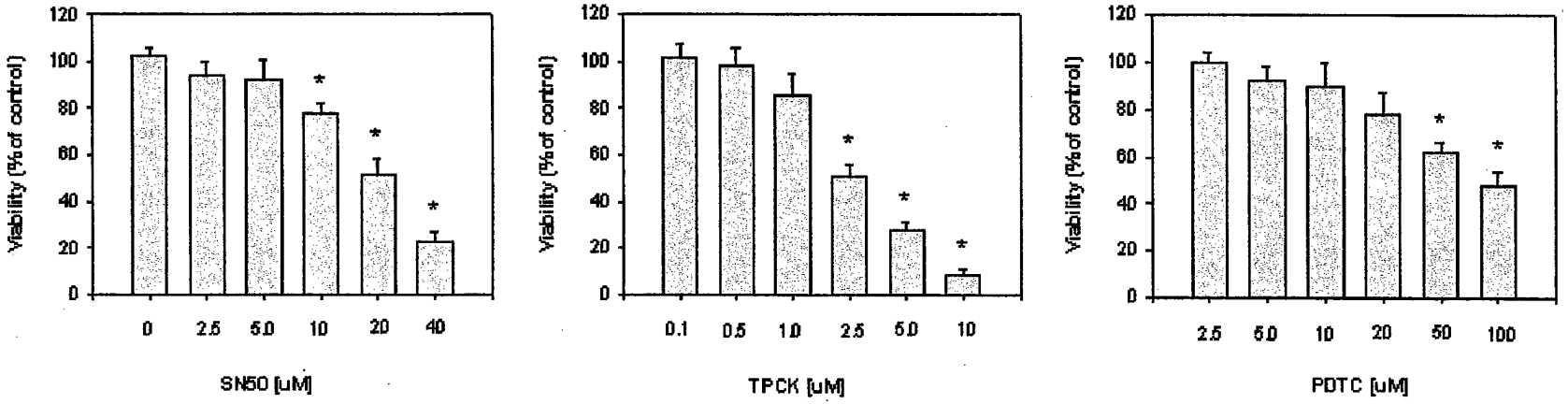
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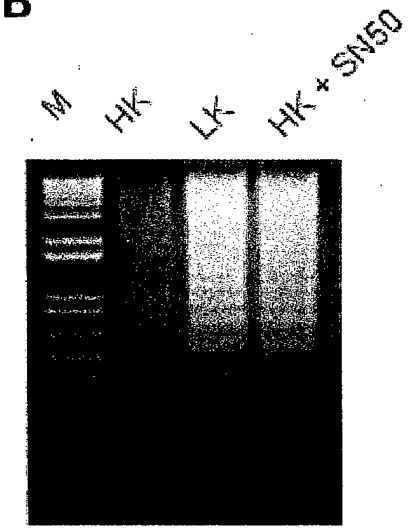
B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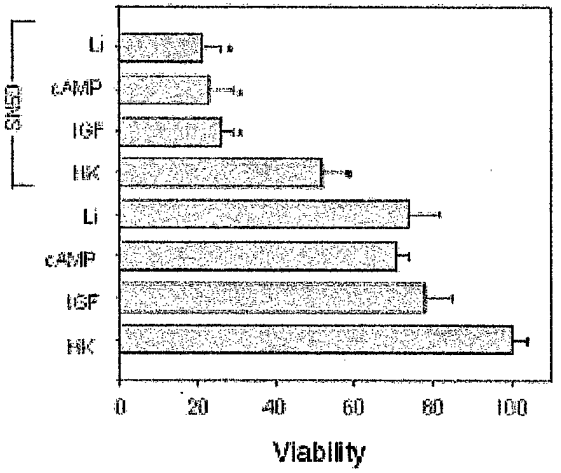
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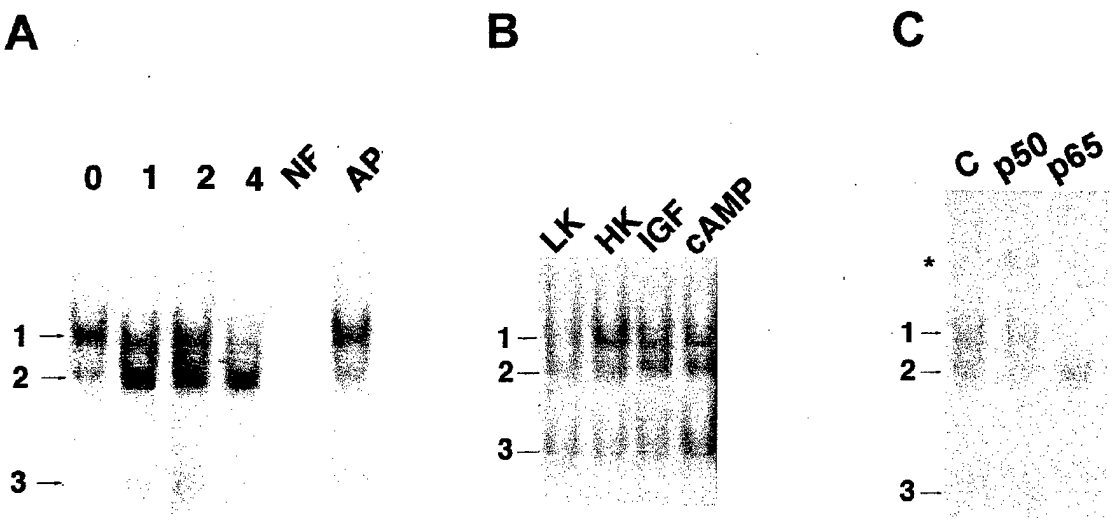
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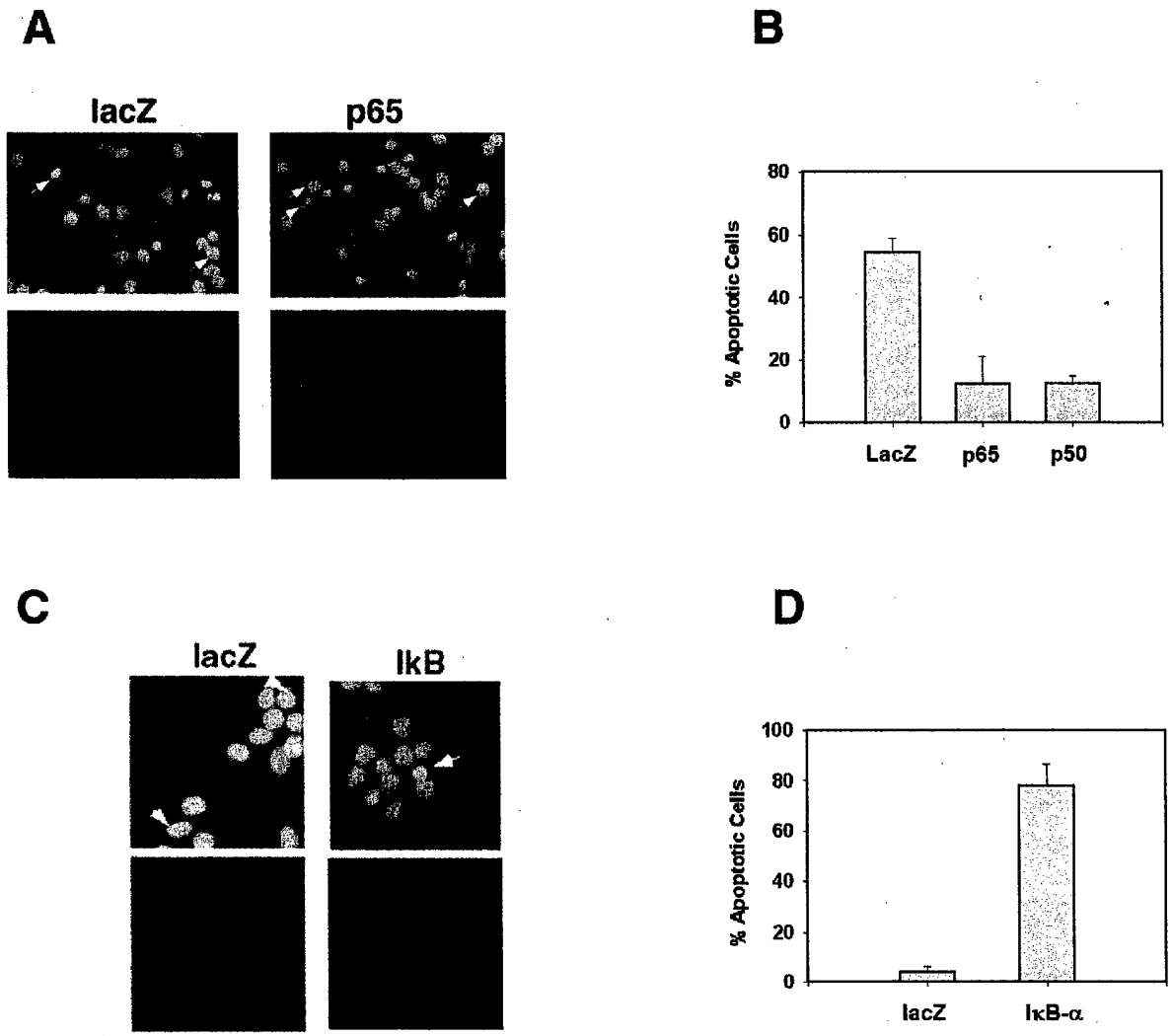
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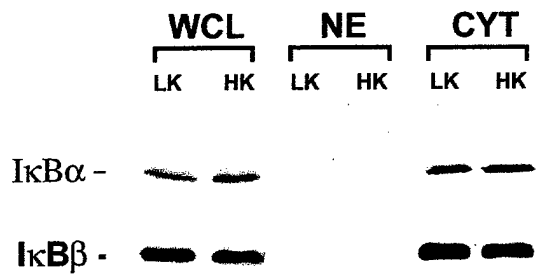
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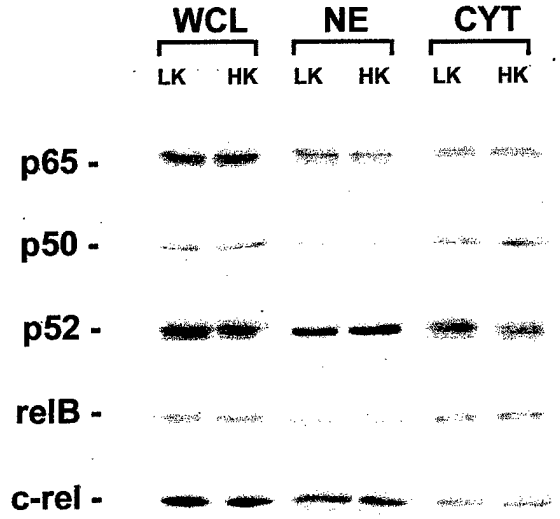
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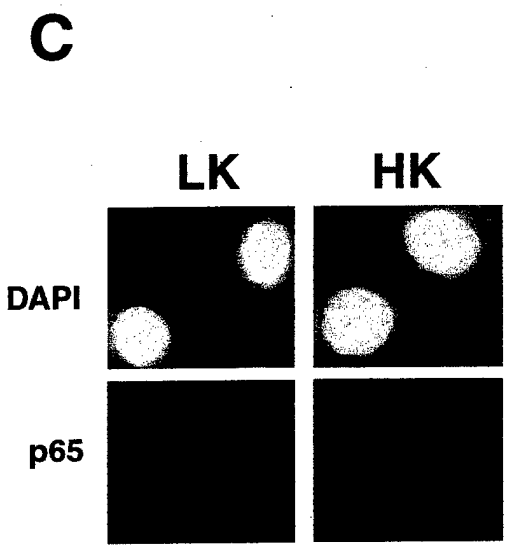
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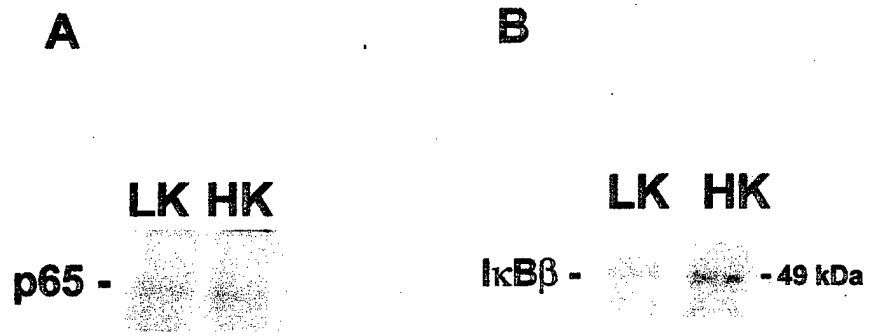
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